RESEARCH ARTICLE

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Oncolytic Newcastle Disease Virus and Photodynamic Therapy as Dual Approach for Breast Cancer Treatment

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Abstract

Objective: We hypothesized that attacking cancer cells by combining various modes of action can hinder them from taking the chance to evolve resistance to treatment. Incorporation of photodynamic therapy (PDT) with oncolytic virotherapy might be a promising dual approach to cancer treatment. **Methods:** NDV AMHA1 strain as virotherapy in integration with aminolaevulinic acid (ALA) using low power He-Ne laser as PDT in the existing work was examined against breast cancer cells derived from Iraqi cancer patients named (AMJ13). This combination was evaluated using Chou–Talalay analysis. **Results:** The results showed an increased killing rate when using both 0.01 and 0.1 Multiplicity of infection (MOI) of the virus when combined with a dose of 6172.8 photons/gm (ph/gm) of PDT focused on cancer cells. **Conclusion:** integration of the attenuated NDV–AMHA1 strain with photodynamic therapy has a synergistic killing effect on breast cancer cells in vitro, suggesting that this strategy could have clinical application to overcome breast cancer.

Keywords: Aminolaevulinic acid (ALA)- breast cancer cells- He-Ne laser- Photodynamic therapy- virotherapy

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Introduction

Breast cancer is a complex and diverse disease that continues to be a serious health problem around the world, affecting millions of women annually [1]. Annually, there is an incidence of more than 2.3 million breast cancers, making it the most prevalent form of cancer in the adult population. Breast cancer ranks as the primary or secondary cause of female cancer fatalities in 95% of countries. However, breast cancer survival rates of approximately 80% of deaths are concentrated in low- and middle-income countries [2]. Iraqi cancer statistics for breast cancer taken from the Iraqi cancer registry, Ministry of Health shows that the highest percentage and incidence rate of the top ten cancers in Iraq was breast cancer [3]. The primary therapeutic approaches for estrogen progesterone negative breast cancer cells include surgery and chemotherapy; however, it is still difficult to treat due to its genetic heterogeneity, low cell differentiation, high malignancy, absence of molecular targets, rapid metastasis, and resistance to chemotherapy [4]. AMJ13 is a hormone receptor-negative breast cancer cell line that is derived from an Iraqi female patient [5]. This cell line expresses several aggressive features, such as secreting many angiogenesis factors [6]. This type of tumors is very aggressive and fatal, Weiss and his team found that senior breast cancer patients with ER/PR-negative have a worse prognosis and are more expected to die of the breast cancer than from cardiovascular disease [7]. Furthermore, triple negative breast cancers (TNBC) have a more aggressive clinical course with an average of less than 3 years to metastasize, leading to death within 5 years [8]. TNBC have this poor prognosis due to chemoresistance and radiotherapy insensitivity [9]. Therefore, it is necessary to look for unconventional methods of breast cancer therapy. Photodynamic therapy (PDT) is an FDA cancer treatment modality that is a light-based treatment that combines physical steps, photosensitizer (PS) stimulation by light, and the photochemical reactions of PS, after excitation, with cellular substrates or molecular oxygen [10]. These two steps lead to the formation of reactive oxygen species (ROS) and eventually to the death of many types of cancer because the cell membrane is ruptured and cell death by necrosis or apoptosis [11, 12]. Photosensitizers (PSs) are ineffective materials formed in host and tumor cells and must be activated via light absorption to obtain a therapeutic photodynamic effect [13]. In mitochondria, the endogenous metabolite ALA is normally formed from succinyl-CoA and glycine. It is not a photosensitizer itself. In fact, the integration of ALA molecules (eight)

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produces protoporphyrin IX (PpIX), which leads to the accumulation of heme utilizing the activity of the rate-limiting enzyme ferrochelatase [14]. PpIX is an effective photosensitizer that is commonly utilized in photodynamic therapy (PDT). Cells with elevated metabolic activity, such as tumors, preferentially absorb ALA [15]. Rather than the formation of heme, the conversion of ALA in these cells leads to the aggregation of protoporphyrin IX (PPIX) due to the lack of ferrochelatase [16]. On the other hand, virotherapy, which utilizes safe viruses to attack cancerous cells, is a promising biological approach for more efficient treatment [17]. With different mechanisms of action, virotherapy causes cancer cell death through direct replication, leading to cell lysis and apoptosis [18]. Newcastle disease virus is a natural oncolytic virus that has been shown to be safe and promising for breast cancer therapy [19].

The Iraqi AMHA1 strain of this virus has been demonstrated to be oncolytic and can destroy cancer cells with excellent safety in vivo [20]. NDV selectively replicates in human cancerous cells but not normal cells and activating apoptotic cell death via DNA fragmentation [21]. NDV induces the intrinsic pathway of apoptosis in vitro, and both intrinsic and extrinsic pathways in vivo [22]. Furthermore, NDV AMHA1 was shown to induce metabolic arrest in breast cancer cells leading to death [23]. Many researchers have shown that tumors that do not respond to monotherapy can be treated by therapy with a combination. This strategy involves various techniques to prevent cells from having enough period to develop resistance to treatment [9]. Virotherapeutic therapy was found to work very well in combination therapy as breast cancer therapy [24]. Newcastle disease virus (NDV) showed a very active effect against tumor cells when integrated with different types of chemotherapies such as 5-fluorouracil, methotrexate rituximab and doxorubicin [25, 26]. The purpose of the present work is to evaluate the combined killing effect of NDV oncolytic virotherapy and ALA-based photodynamic therapy against breast cancer, exploring their potential synergy in the killing of cancer cells in vitro.

Materials and Methods

Cell line and culture medium

The human AMJ13 cell line (breast cancer) cell line was obtained from the Iraqi Center for Cancer and Medical Genetics Research ICCMGR-Iraq-Baghdad. Cells were kept in RPMI-1640 medium containing sodium bicarbonate (2.0 g/L) and L-glutamine (0.3 g/L) and supplemented with 10% fetal bovine serum (FBS) and 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin All materials were provided by (Sigma-Aldrich-Germany). Cells were grown to near confluence and then subcultured with Trypsin-EDTA (US biological, Salem, MA, USA) at 50-60 % confluence twice a week and incubated at 37 ° C. Subsequently, cells were seeded at 1 $\times 10^4$ cells per well in 96-well culture plates and incubated in an incubator at 37 °C for 24 h.

Photosensitizer and Laser Irradiation

Aminolaevulinic acid (ALA) was purchased from Santa Cruz Biotechnology (Texas, USA). Phosphate buffer saline (PBS) was used to dissolve ALA in a dark room according to the manufacturer's instructions. The PH was adjusted to 7.0 using NaOH (1M). A stock solution of 60 mM was prepared. The helium-neon atomic gas laser (Model DL30, LG Lasers) was used as the laser source in this study. The wavelength of light emitted from this source was 632.8 nm (red light) and the output power was equal to 20 mW (milliwatt) as a continuous wave. The laser doses used were 10.8 J/cm^2 or 16.2 J/ cm^2. To calculate the photodynamic dose, we used the formula:

Photodynamic Dose (PD) = $\int_0^t \varepsilon * c * \varphi(t') * \rho * h\nu \, dt'$

Where:

PD: Photodynamic Dose (J/cm^3)

ε: Molar extinction coefficient of the photosensitizer (cm^{-1} M^{-1})

c: Drug (photosensitizer) concentration in tissue (mol/cm^3)

φ(t'): Light fluence rate at time t' (W/cm^2)

ρ: Tissue density (g/cm^3)

hν: Energy of a photon (J/photon)

t: Irradiation time (s)

dt: Infinitesimal increment of time (s) [27].

For example, when we used 2mM of ALA with 16.2 J/cm^2 , the photodynamic dose was 6172 photons/gm.

Exposure of breast cancer cells to laser

After 24 h of incubation time of cells seeded in a 96-well plate, cells were incubated with freshly prepared 1 or 2 mM of ALA for 4 hours at 37 °C. Subsequently, the medium was changed with fresh SFM after washing with physiological saline. The laser applications occurred after that. Neither ALA nor irradiation was used with control cells that were incubated with fresh medium. Irradiation with laser light was carried out using the helium-neon atomic gas laser lamp, the light was focused from the top of the wells to ensure a symmetric delivery to each well. A power meter was used to calibrate laser power and the time of irradiation was adjusted to acquire a power density of 10.8 and 16.2 J/cm^2. After that, the virus at MOI (0.1,0.01, 0.001) was added and incubated for two hours at room temperature; then, the cells were incubated at 37 °C for 72 hours. The cell death rate was determined by the MTT assay. The work was carried out under minimal ambient lighting.

Newcastle disease virus

The attenuated Iraqi strain of NDV (Iraq/Najaf/ICCMGR/2013) [28] was supplemented by the Cell Bank Unit/Experimental Therapy Department/Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). This virus was reproduced in embryonated chicken eggs (Al-Kindi Company, Baghdad, Iraq). The 50% Tissue Culture Infectious Dose (TCID50) titration was determined for the virus according to the standard procedure [25]. Two hours of incubation of cells with the virus was achieved at room temperature to allow viral attachment and penetration. Subsequently, cells were washed (with PBS) and then an MTT assay was used to measure viability after 72 h

of infection.

Cytotoxicity (MTT tetrazolium assay)

The methyl thiazolyl tetrazolium (MTT) assay was used to measure cytotoxicity, 20 µl of (5X) MTT was added to each well and incubated for 3 h at 37 °C. In each well, (200 µl) Dimethyl sulfoxide (DMSO) was added, and the plate was shaken for 15 m. To measure the absorbance, a microplate reader (FLUOstar, Optima) was used at 492 nm. The measured values were calculated as means. To calculate the percentage of cell killing rate, the following formula was used:

Cell killing rate (%) = [OD of control cells - OD of treated cells)/OD of control cells] x100% (13).

Statistical analysis

The MTT assay was statistically analyzed using the one-way analysis of variance test (ANOVA) in GraphPad Prism (GraphPad Software, Inc. San Diego, California). The standard deviation of the mean was considered significant at P = 0.05.

Statistical analyses for in vivo study were performed with GraphPad Prism (GraphPad Software Inc.). one-way ANOVA analysis of variance tests were used for statistical comparison between three or more groups. Data in graphs are shown as mean± S.D.

To determine synergism, NDV and PDT were studied as a non-constant ratio. To analyze the combination of NDV and PDT, Chou-Talalay combination indices (CI) were calculated using CompuSyn software (CompuSyn, Inc., Paramus, NJ, USA). The non-fixed ratios of NDV and PDT as well as mutually exclusive equations were used to determine the CIs. A CI between 0.9 and 1.1 was considered additive, whereas CI<0.9 and CI>1.1 indicated synergism and antagonism, respectively [29].

Results

Virus Cytotoxicity

Viral attachment and penetration were achieved by incubation of cells with virus for 2 h at room temperature. Cytotoxicity (CT %) of different MOIs of NDV of the virus was evaluated using the MTT cytotoxicity assay. Through our findings, an increase in MOI of the virus was shown to improve growth inhibition or increase cytotoxicity. Furthermore, there was a significant difference between the optical density of 0.001 MOI and a highly significant difference of 0.01 and 0.1 MOI compared to the control (cells without virus) as shown in Figure (1, A).

ALA / Laser Cytotoxicity

The effect of using the ALA precursor with low-dose laser on cytotoxicity was evaluated using different concentrations of ALA and laser doses. The results have shown that the use of 1 mM ALA with 16.2 J/cm^2 (3164.2 ph/gm) has a significant cytotoxic effect against the AMJ13 cell line (P-value < 0.0061). Furthermore, increasing the concentration of ALA to 2 mM with 10.8 or 16.2 J/cm^2 (5092 ph/gm) has a highly significant difference in cytotoxicity against the AMJ13 cell line. Figure 2 revealed this effect (P value < 0.0001****).

Determination of IC_{50}

To evaluate the effect of each treatment with NDV or ALA/Laser on cell proliferation, the value of the half maximum inhibitory concentration (IC_{50}) was measured for each of them. IC_{50} was estimated by plotting x-y and fitting the data with a straight line. The IC_{50} value was then estimated using the fitted line, that is:

$Y = a * X + b$, $IC_{50} = (0.5 - b)/a$

Or by:

$Y=Bottom + (Top-Bottom)/(1+10^{((X-LogIC_{50}))})$

The results showed that 0.0069 MOI viruses were needed to kill the AMJ13 cell line, while a 1772 ph / g photothermic dose was needed to kill AMJ13 Iraqi breast cancer cells, Figure (3, A & B). Therefore, doses related to IC_{50} of ALA / Laser and NDV were chosen for the combination study.

The synergistic effect of NDV and ALA/Laser

To detect the cytotoxic effect of NDV and ALA/Laser in synergistic remediation, a 96-well plate (Franklin

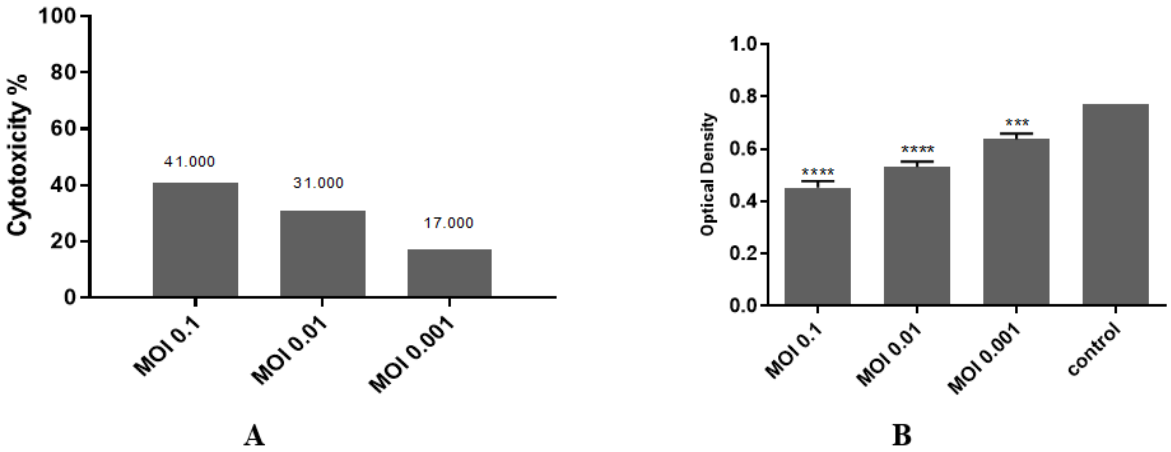

Figure 1. A: Cytotoxicity of the treated AMJ13 cell line with (0.1,0.01, 0.001 MOI) of NDV. B: Mean optical density (mean ± SD), (P< 0.0001****).

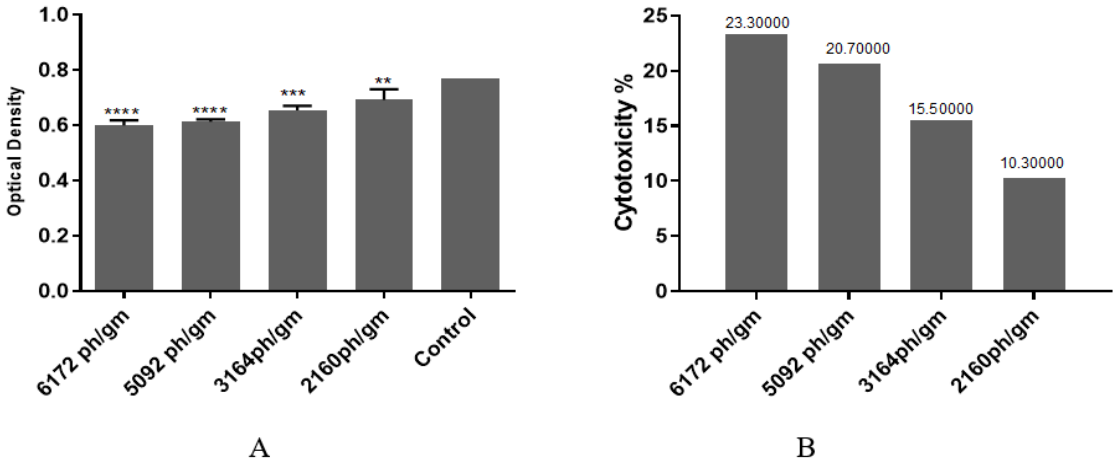

Figure 2. A, mean optical density (mean ± SD), B; cytotoxicity of the treated AMJ13 cell line with (2 or 1 mM) of ALA with laser doses of 16.2 or 10.8 J/cm^2 to obtain 6172, 5092, 3164 or 2160 ph / gm, respectively (P< 0.0001****).

Table 1. Combination Index Data, each Dose of NDV with ALA / Laser is Shown.

NDV Dose NDV (MOI)	Photodynamic dose of lase (ph/g)	Effects	CI
0.1	6172	0.48	0.72231
0.1	5092	0.46	0.84399
0.1	3164	0.43	1.10263
0.1	2160	0.42	1.20107
0.01	6172	0.41	0.52733
0.01	5092	0.41	0.45685
0.01	3164	0.43	0.28013
0.01	2160	0.37	0.40429
0.001	6172	0.2	1.82015
0.001	5092	0.19	1.83736
0.001	3164	0.18	1.68773
0.001	2160	0.18	1.45655

Lakes, USA) was used to detect the MTT cell viability assay. AMJ13 cells were seeded at 1×104 cells/well. After achieving a 60-70% confluence monolayer (or after 24 h.), the cells were exposed to ALA/laser and incubated with NDV (at MOI 0.1, 0.01, 0.001). Two concentrations of ALA, 2 or 1 mM, with two laser doses (16.2 or 10.8 J/cm^2) to obtain 6172.8, 5092, 3164.2 or 2160 ph / gm, respectively. After 72 h of infection, cell viability was measured. The assay was performed in triplicate. Boosted cytotoxicity was achieved for the synergism of NDV and ALA/Laser at generality doses.

Combination Cytotoxicity Assays and Chou-Talalay Analysis

To determine the synergistic effect, we studied NDV and ALA/Laser (PDT) as a nonconstant ratio. To analyze the combination of NDV and ALA/Laser, Chou–Talalay combination indices (CI) were detected using CompuSyn software (Combo Syn, Inc., Paramus, NJ, USA). NDV and photosensitizer/Laser, as non-fixed ratios, as well as mutually exclusive equations, were utilized to determine the CIs. A CI between 0.9 and 1.1 is considered additive, while CI < 0.9 and CI > 1.1 indicate synergism and antagonism, respectively [30]. In this combined therapy, the killing rate increased when 0.1 MOI of the virus (in comparison of 0.01 or 0.001) was combined with 2

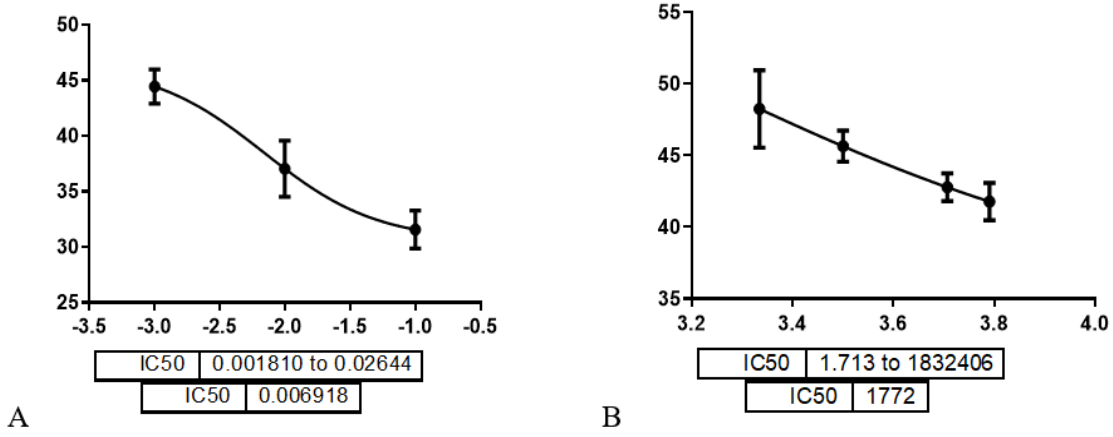

Figure 3. A, IC$_{50}$ to exposure of NDV to the cell line, B; IC$_{50}$ to exposure of ALA/Laser to the cell line using GraphPad Prism software.

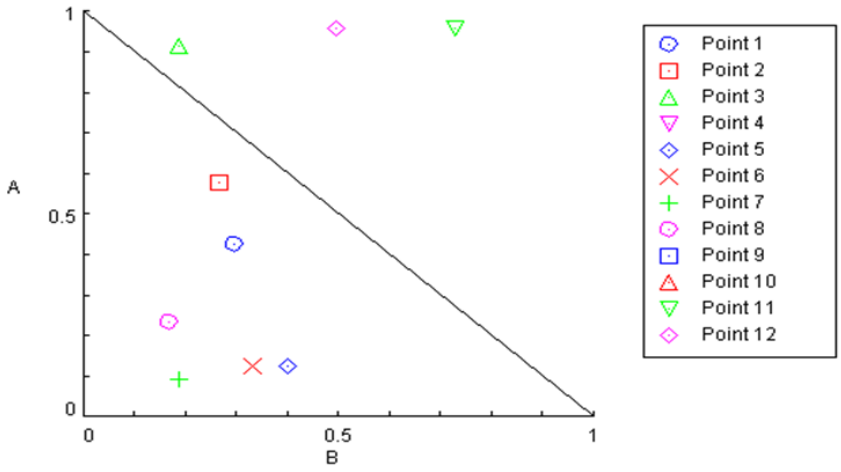

Figure 4. Combination Cytotoxicity in the AMJ13 Cell Line. According to the Isobologram analysis, high doses (CI value < 1) represent synergism between NDV and phototherapy, and lower doses (CI value > 1) represent antagonism between agents. The points that are extremely antagonized will not be able to be included as their value is above 2 and will not show in this figure.

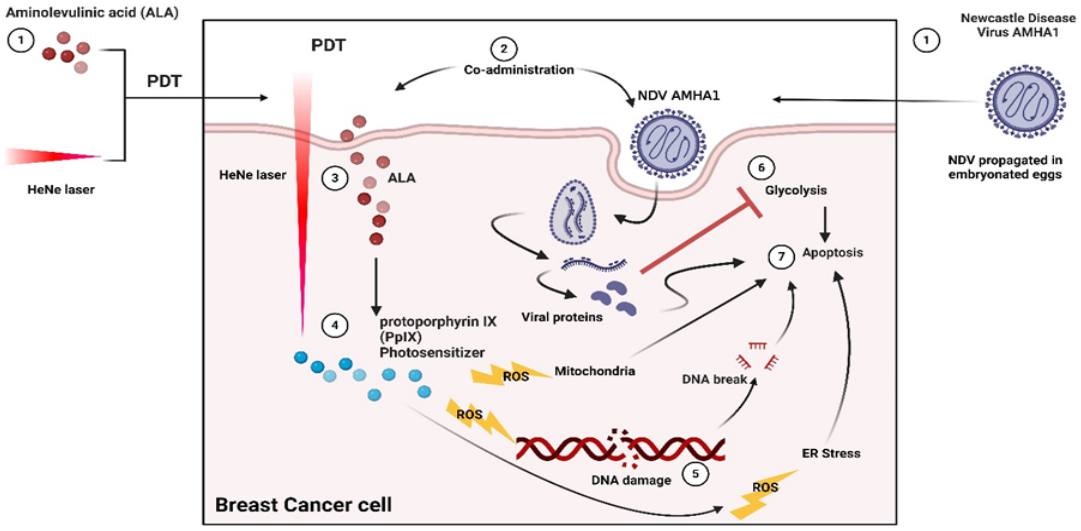

Figure 5. The Proposed *in vitro* Mechanism of Action in which Both Treatment Modalities Induce Apoptosis via Different Modes of Actions Leading to Amplified Results

mM ALA when 16.2 J/cm^2 (6172.8 ph/gm) dose of laser focused on cells. However, no, or very little difference in effect occurred between 6172 or 5092 ph/gm at the same concentration of NDV Figure 4. The combination index data for each dose are shown in Table 1.

Discussion

This work aimed to explore whether we can increase the effect of virotherapy against resistant breast cancer cells by photodynamic therapy. The cell line used AMJ13 is an Iraqi breast cancer patient derived cell line that has higher expression of ABCA1 and ABCA3, which is linked to their resistance to chemotherapy [31]. The current investigation combined two unconventional cancer therapeutics to overcome breast cancer resistance. Photodynamic therapy (PDT) is FDA approved cancer therapy characterized as a minimally invasive anticancer treatment that uses a mix of photosensitizer-mediated cell killing and light irradiation [32]. While oncolytic virotherapy using Newcastle disease virus is promising approach that showing high selectivity and safety with high activity against breast cancer cells [33]. NDV has many characteristics that make it convenient for human use. These characteristics comprise the genomic stability, loss of genetic recombination, absence of antigenic drift, and loss of observed human-to-human transmission [34, 35]. Based on our results with the chemoresistance breast cancer AMJ13 cell line, NDV exhibited anticancer activity, as was shown in previous studies [36, 37]. Furthermore, broad spectrum anticancer action was noticed such as anti-lymphoma activity has been shown by NDV [38, 39]. Also, 700-fold higher electively against pancreatic cancer than normal cells was shown by [40].

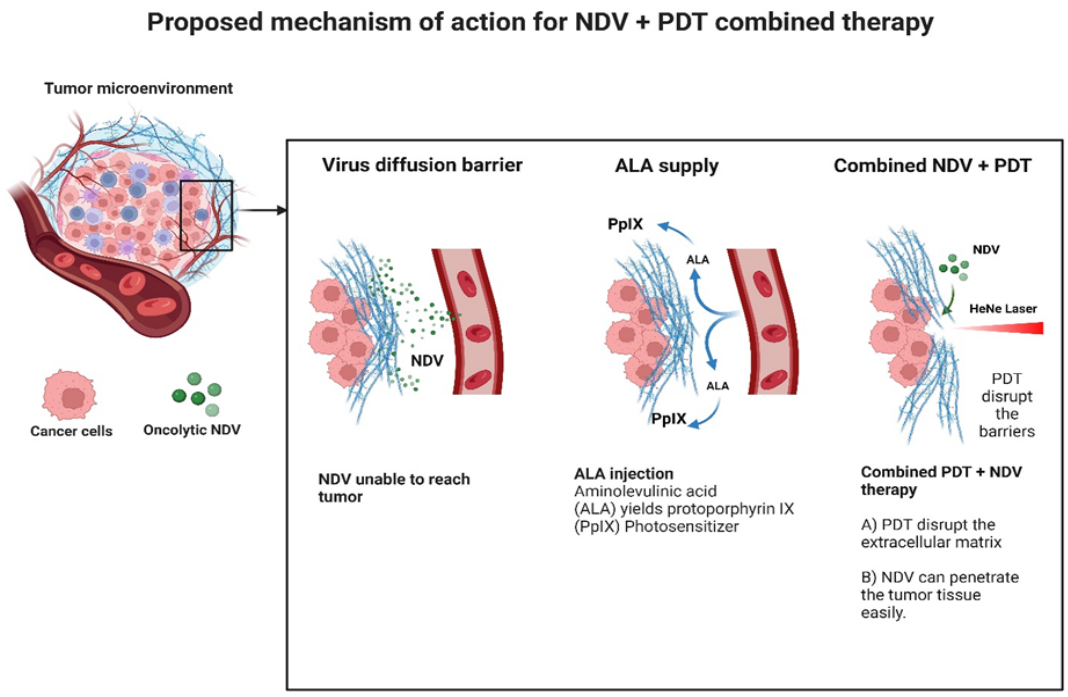

Figure 6. We also Hypothesized an *in vivo* Mechanism of Action that may Work for Future Experiments *in vivo* where the PDT can Help to Rupture the Blood Barriers, Leading to Better Virus Distribution in the Tumor Tissue.

Furthermore, in several studies, the combination of oncolytic NDV with the standard chemotherapeutic agent with observed increased cytotoxicity, indicating that NDV works well in combination therapy leading to synergistic anticancer action [41, 42]. According to Chou–Talalay analysis, co-administration of NDV and photodynamic therapy increased the killing effect compared to NDV alone. However, the mechanism of synergistic activity is not clear. In addition to the suggestion that PDT could increase viral replication, the synergistic effect could be due to the increased anticancer activity by combined action, which can lead to increased cell sensitivity to PDT or virotherapy. The activity of this combination therapy might enhanced the action of each individual treatment, for example, photodynamic therapy induce apoptosis [43], or due to an augmented decrease in the amount of microsomal protein Erp72 and an up-regulation of calmodulin and HSP-60 level [44]. Not only the anti-apoptotic proteins of the Bcl-2 family (Bcl-2 and Bcl-xL) might have declined, but also the pro-apoptotic protein of the same family Bax [44] due to the use of this technique. Furthermore, mitochondria may be sensitive to this type of therapy via rapid release of cytochrome C or by up-regulation of glycolytic phosphorylation [45]. On the other hand, oncolytic NDV also confirmed to induce apoptosis via induction of both intrinsic and extrinsic pathway of apoptosis and involvement of caspase independent pathway of apoptosis [39, 22]. Our team discovered that the oncolytic AMHA1 NDV strain induces inhibition of the glycolysis pathway leading to starvation of breast cancer cells and apoptosis [37]. The proposed in vitro mechanism of action is summarized in Figure 5 in which both treatment modalities induce apoptosis through different modes of action leading to amplified results. While this mechanism of action is limited to our in vitro study, we also hypothesized an in vivo mechanism of action that may work for future experiments in vivo where the PDT can help to rupture the blood barriers leading to better virus distribution in tumor tissue as summarized in Figure 6.

In conclusions, an attenuated non-pathogenic AMHA1 NDV combined with photodynamic therapy has a synergistic impact in vitro on chemoresistant breast cancer cells, suggesting that this new treatment combination could have a remarkable clinical application to overcome aggressive breast cancer.

Author Contribution Statement

A.M. Al-Shammari and A.S.K. Al-Khafaji made substantial contributions to the conception and design of the research project, analysis, and interpretation of data, generated the datasets and drafted the work. M.I. Salman contributed to the acquisition, analysis, and interpretation of literature data, and revised the manuscript. H.A. Hassan performed laboratory work and contributed to the acquisition of literature data, provided interpretation, and revised the manuscript. A.M. Al-Shammari sufficiently participated in the acquisition, performed data analysis, interpretation of the data and revised the manuscript. All the authors have read and approved the final version of the manuscript for publication.

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Study Approval

The study proposal was reviewed and approved by the scientific committee of the National Cancer Research Centre, University of Baghdad, Iraq and the scientific committee of the Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University

Ethics Approval

No ethical approval for the present study was needed as no involvement of human samples or laboratory animals.

Conflict of Interest

The authors declare that they have no conflict of interests.

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